Reprinted from JOURNAL OF BACTERIOLOGY
Vol. 68, No. 4, pp. 419-429, October, 1954
Printed in U.S.A.

#73

ON THE ORIGIN OF THE CARBON IN THE INDUCED SYNTHESIS β-GALACTOSIDASE IN ESCHERICHIA COLI[1,2]

B. ROTMAN[3] AND S. SPIEGELMAN

Department of Bacteriology, University of Illinois, Urbana, Illinois

Received for publication March 29, 1954

Recently reported experiments have made it necessary to assume that the induced synthesis of enzymes in microorganisms is coupled mandatorily to the utilization of free amino acids. Investigations (Halvorson and Spiegelman, 1952) with yeast have shown that analogues of amino acids which prevent incorporation from the free amino acid pool into protein also suppress the induced formation of α-glucosidase. Furthermore, a complete parallelism between α-glucosidase synthesizing capacity and the available internal supply of free amino acids was demonstrated by means of depletion and replenishment cycles (Halvorson and Spiegelman, 1953*a*). Finally, employing the same system, it was possible to exhibit (Halvorson and Spiegelman, 1953*a*) a net increased utilization of the internal free amino acids as a consequence of the induction of enzyme synthesis in cells suspended in a nitrogen-free medium. The generality of the findings with amino acid analogues was extended by the independently performed experiments of Lee and Williams (1952). These authors demonstrated that the administration of ethionine to the intact rat prevented the induced formation of tryptophan peroxidase.

Monod *et al.* (1952) studied the formation of β-galactosidase in a series of amino acid auxotrophic mutants of *Escherichia coli*. In no case was enzyme synthesis observed in the absence of the particular amino acid required. Rickenberg *et al.* (1953) report experiments along similar lines in which β-galactosidase formation was followed in mutant strains of *E. coli*, strain K-12. Analogous results also were obtained in the induction of a myoinositol degradation system in *Aerobacter acrogenes* (Ushiba and Magasanik, 1952).

The data cited would appear to eliminate any mechanism of enzyme synthesis which involves an amino acid independent transformation of a preexistent complex precursor into active enzyme. They do not, however, make untenable, or distinguish between, the following two possibilities for enzyme synthesis:

(a) Precursor and free amino acids → Active enzymes.

(b) Free amino acids → Active enzyme.

It is the purpose of the present paper to offer data relevant to this issue. The most obvious experimental approach aimed at a decision between the above two alternatives would appear to be the use of isotopic labels. Thus, the induction of enzyme synthesis in uniformly labeled cells suspended in unlabeled medium should provide the necessary data.

It is evident from the type of experiment contemplated that the presence of a large internal free amino acid pool, such as exists in the yeasts and many gram positive bacteria, would introduce a complication which could well make the data uninterpretable. To avoid this complication, attention was turned to enzyme formation in gram negative microorganisms which have been shown (Taylor, 1947) to lack detectable free amino pools. The system selected for study was the β-galactosidase of *E. coli*. The choice of this particular enzyme was dictated by a variety of reasons. Lederberg (1950) devised a convenient and accurate assay of its activity by employing the synthetic chromogenic substrate *o*-nitrophenyl-β-D-galactoside. In addition, the intensive investigations to which this system in *E. coli* has been subjected in recent years have elucidated not only purely enzymatic properties (Cohn and Monod, 1951; Kuby and Lardy, 1953; Lester and

[1] This research was aided by a grant from the National Cancer Institute of the National Institutes of Health and by a contract between the Office of Naval Research, Department of the Navy, and the University of Illinois, NR 135-136.

[2] A summary of the present results was presented at the 1953 meeting of the Society of American Bacteriologists (Bacteriol. Proc., 1953, p. 92).

[3] Present address: Enzyme Institute, Madison, Wisconsin.

Bonner, 1952; Lester, 1952) but also have provided detailed information on the genetic (Lederberg, 1952), immunological (Cohn and Torriani, 1952, 1953), and the specificity aspects of the inductive process (Monod *et al.*, 1951; Koppel *et al.*, 1953).

The experiments to be described in the present paper employed C^{14} as the isotopic label. The results obtained lead to the conclusion that there is little or no preformed complex precursor which is converted into active enzyme. Analogous findings and similar conclusions were arrived at simultaneously by Cohen and Hogness (for a preliminary account, see Monod and Cohn, 1953). These authors employed S^{35} as the isotopic label and predominantly immunological procedures for the isolation of the β-galactosidase.

MATERIALS AND METHODS

Organism used and conditions of growth. Strain ML of *E. coli* was used. It was obtained through the courtesy of Dr. S. E. Luria. Stock cultures on synthetic medium served as the source material for the preparation of experimental suspensions. Unless otherwise specified, a modification (reduction of the $CaCl_2$ by one-half) of the synthetic medium (M-56) described by Monod *et al.* (1951) was used. A suitable carbon source was added to the above medium and the cultures incubated at 37 C with vigorous aeration.

Chemical and radioactive reagents. ortho-Nitrophenyl-β-D-galactoside (ONPG) was synthesized according to the method described by Seidman and Link (1950). Melibiose was prepared from raffinose according to the procedure of Hudson and Harding (1915). Uniformly C^{14} labeled sucrose was prepared from $C^{14}O_2$ by the use of leaves of *Canna indicus* (Gibbs *et al.*, 1952). The C^{14}-sucrose was converted to lactate by carrying out a fermentation with *Streptococcus faecalis*. L-Lactic acid (Pfanstiehl) was employed as a carrier in the preparation and purification (Wood *et al.*, 1945) of the labeled lactate.

All the water used in the present investigations was double distilled from a glass still.

Analytical procedures. Lactate was analyzed by the method of Barker and Summerson (1941) and nitrogen by the nesslerization procedure of Lanni *et al.* (1950). In the latter, color was measured at 500 mμ in a Beckman spectrophotometer using 0.5 ml cells. For preliminary survey experiments, the micro-Folin method (Lowry *et al.*, 1951) for the determination of protein was used.

Radioactivity measurements were made with a "Nuclear" Q-gas flow counter which possessed a background count in the neighborhood of 15 to 20 counts per min. The specific radioactivities and, hence, the amounts required for measurement were such that corrections for self adsorption were only necessary occasionally. Counting was continued until between 1,500 and 2,000 counts had accumulated. In certain critical cases, longer counting times were used to increase the accuracy.

To obtain an accurate estimate of the specific radioactivity of the protein in the eluates from the starch column, it was necessary to remove adventitious nonprotein carbon. Purification of the protein was achieved by precipitation with trichloracetic acid and subsequent extraction with hot trichloracetic acid followed by further extractions with ether-methanol mixtures. A typical purification of this nature may be detailed as follows: To two ml of an eluate from a starch column is added 0.3 ml of a 50 per cent trichloracetic acid. The resulting precipitate is spun down, and 5 ml of 5 per cent trichloracetic acid added, allowed to extract for 25 hr at room temperature, and then brought to 90 C and held for 10 min. The precipitates are centrifuged then and extracted twice with 5 ml of a 1:1 methanol-ether mixture. The residue is collected by centrifugation and dissolved in one ml of 0.5 N NaOH which subsequently is neutralized with HCl.

Enzyme was assayed by a modification of the procedure suggested by Lederberg (1950). It was found that, whereas freshly recrystallized *o*-nitrophenyl-β-D-galactoside was hydrolyzed in the presence of enzyme linearly with time, divergencies from linearity were obtained with older preparations. It was established empirically that the incorporation of yeast extract in the assay preparation counteracted this effect. To avoid the necessity of continually recrystallizing the reagent, yeast extract was included. The latter was prepared from pressed baker's yeast (Fleischmann's) in the following manner: 54 g of yeast are suspended in 20 ml of H_2O, autoclaved at 15 lb and 120 C for 0.5 hr. The resultant extract is centrifuged, the supernate brought to a boil, treated with decolorizing carbon, and filtered. The filtrate is stored in the deep freeze.

The enzyme reaction was run at 40 C in the presence of 0.001 M *o*-nitrophenyl-β-D-galactoside dissolved in a mixture of NaH_2PO_4 and Na_2HPO_4 to yield a solution at pH 7.5, 0.1 N with respect to Na, and containing 0.04 ml of yeast extract per ml. The development of color was followed by means of a Klett-Summerson colorimeter using filter no. 42a. One unit of enzyme activity is represented by the hydrolysis of one mμ mole of *o*-nitrophenyl-β-D-galactoside per minute under the above conditions. Specific activity is reported in units per μg of nitrogen.

Preparation of purified antiserum. The methods employed for the preparation and purification of anti-β-galactosidase antiserum were derived from the investigations of Cohn and Torriani (1952). β-Galactosidase purified as described below was used as the antigen. Approximately 0.2 mg of purified antigen was injected into each rabbit with mineral oil as an adjuvant (Freund and Bonanto, 1944). Sera were collected, pooled, and the γ_2-globulin fraction isolated by ethanol fractionation (Nichol and Deutsch, 1948). Use of such preparations avoids the nonspecific precipitation which often occurs on adding bacterial extracts to whole serum. The conditions described by Cohn (1952) and Cohn and Torriani (1952) for obtaining specific precipitations of β-galactosidase with antiserum were followed.

Purification of β-galactosidase. The purpose of the experiments to be described demanded a procedure for the isolation of pure enzyme from small amounts of material which would be efficient in the yield of final product and lead to a minimal amount of inactivation. The latter was necessary to avoid the complications in specific activity determinations which would derive from the presence of enzyme protein not identifiable in terms of enzyme activity. It was evident that the classical methods of enzyme separations used by previous authors (Monod and Cohn, 1952; Kuby and Lardy, 1953; Lester, 1952) for the purification of β-galactosidase did not satisfy either of these criteria and could not be used without drastic modification. Other methods were sought and particular attention was paid to the potentialities of electrophoretic separation of protein on filter paper (Durrum, 1950; Cremer and Tiselius, 1950; Kunkel and Tiselius, 1951). Despite a considerable amount of effort it was found impossible to adapt this procedure for the purposes at hand. The amount of material which could be accommodated in one run, even using piles of paper strips, was too small for feasibility. In addition, the resolution of the enzyme from other contaminating proteins was not sufficiently sharp on any filter paper types available for trial. Attention, therefore, was turned to other solid material which could act as a suitable neutral support. While the present investigations were in progress, Kunkel and Slater (1952) reported briefly on an extension of the paper strip method. They came to the conclusion that starch was the most desirable material to use, based both on low adsorbing capacity for protein and minimal electroosmotic flow of water. In agreement with Kunkel and Slater (1952) the present authors found that insoluble potato starch yielded the best results of all the materials tested. It showed no tendency to adsorb specifically either the β-galactosidase or the other proteins present in extracts of *E. coli*. This latter property is of the utmost importance if trailing of proteins in the column is to be avoided.

The insoluble potato starch used was obtained from E. H. Sargent and Co. It was found necessary to remove contaminating nitrogenous material from all commercial potato starches examined. This was accomplished by washing the starch several times with three times its volume of 0.05 N alkali. The alkali was removed with water, which then was followed by several washings with the buffer to be employed. The starch was stored under buffer in the cold at 4 C. Starch for the solidification of input material was obtained by drying starch following the water wash.

The steps followed in the isolation of enzyme may be summarized briefly as follows:

(1) Subsequent to induction of enzyme, cells are harvested and washed with H_2O followed by centrifugation. All the subsequent steps were carried out at 4 C.

(2) Cells are resuspended to a density of approximately 50 mg wet weight per ml and subjected to sonic disintegration in a 10 kilocycle Raytheon oscillator (70 v at approximately one ampere) for 15 to 20 min.

(3) The resultant extract is centrifuged for 45 minutes at 15,200 G in a Sorvall SS-1 centrifuge. The pellet is discarded.

(4) The enzyme in the supernate of step 3 is concentrated by ammonium sulfate fractionation

(65 per cent) in the cold. The precipitate is separated by centrifugation, redissolved in M/10 phosphate buffer at pH 7.5, and the ammonium sulfate (52 per cent) precipitation repeated. The second precipitate is redissolved in H_2O, and the pH is adjusted to neutrality. In this manner, an increase of between five and tenfold in enzyme concentration can be achieved along with the removal of a large portion of the nonprotein material. The purification attained by these steps rarely exceeds 3×. Nevertheless, such concentration steps are necessary whenever it is desirable to place reasonably large amounts of active enzyme on the columns.

(5) The residual ammonium sulfate in the enzyme solution which attends carrying out step 4 was removed by dialysis against water. This removal was necessary to avoid trailing of protein during the course of the electrophoretic separation.

(6) In the present investigation, starch columns 5 cm in width generally were used in the first runs. The thick starch slurry was poured in lucite troughs 48 cm long and 1.5 cm high. Excess moisture was removed by strips of heavy blotting paper. The columns are allowed to sit in contact with the electrode vessels for a period of two hours to permit equilibration. Such columns can easily accommodate 7 ml (equivalent to between 50 and 70 ml of original extract) of the dialyzed extracts obtained in step 5 if two cm trenches are cut out in the preformed column (close to negative cell). Dry starch is added to the protein solution until a consistency is reached which is equivalent to that of the column. The contents are placed in the initial trench and further solidification achieved by adding more dry starch. The columns then are covered with lucite tops and placed between two electrode vessels similar in construction to those described by Kunkel and Tiselius (1951). Contact between the ends of the starch column and the electrode vessels is accomplished by means of plastic sponges sitting in the forward compartments of the vessels. The entire apparatus, other than the constant voltage supply, is run in a cold room held at 4 C. Efficient cooling of the column is effected by having it in contact, both from above and below, with lead bricks such as are commonly used for radioactive shielding. The voltage applied ranged between 300 to 450 v yielding an amperage between 6 to 25 milliamperes, depending on the cross-sectional area of the column. We have employed routinely 0.025 M Na_2HPO_4·$10H_2O$ (Merck) as the buffer adjusted to the desired pH at 4 C with HCl. This buffer can be used conveniently at both pH 8.4 and pH 6.5.

The column is allowed to develop for between 12 to 14 hr, by which time (at 350 v) the β-galactosidase will have moved about 23 cm from the starting position. The position of the enzyme peak can be determined easily in a variety of ways. One method is to remove sample plugs at one cm intervals along the column with the aid of a trocar cut off near the nub and fitted to a syringe. The starch plugs so obtained then are placed in separate tubes and eluted with H_2O or buffer. Enzyme assays and protein determinations can be made then on the eluates. The elution is carried out at 4 C for 40 minutes while tubes are being shaken in an inclined position on a platform shaker. When placed in an upright position the starch in the tubes settles within 20 minutes, permitting the decantation of the eluate. Once the enzyme has been located, the block containing the peak region can be cut out and replaced in a prepared initial trench of a fresh column. This process can be repeated until constant specific activity in the peak region is attained.

Conditions of induction. Enzyme was induced in aerated cultures growing exponentially in a synthetic medium with L-lactate as the sole carbon and energy source. The latter is "neutral" with respect to β-galactosidase synthesis, in the sense of not interfering with it. Melibiose, which is not utilizable by strain ML, was used as the principal inductor to avoid the complications (Monod and Cohn, 1952) which can attend the presence of a metabolizable inductor.

EXPERIMENTAL RESULTS

Purification of enzyme. It is the purpose of the present section to describe some representative details obtained in the course of purifying β-galactosidase from partially induced cells of *E. coli*. The relative efficiency of the starch column procedure may be illustrated by the results observed in the first run with a crude extract in which no attempt at preliminary purification or concentration of enzyme by means of ammonium sulfate fractionation was attempted.

Logarithmically growing cells, suspended in 300 ml of synthetic medium, were incubated in

37 C with aeration in the presence of 0.001 M melibiose as inductor. The induction was stopped after 97,000 units (mμM of *o*-nitrophenyl-β-D-galactoside split per minute) of enzyme had been synthesized. During this same period an increase in cell mass equivalent to 0.37 divisions had occurred. The cells were harvested, washed, and subjected to sonic lysis. The extract was cleared of cell debris by centrifugation for 15 minutes at 15,000 G. The resulting extract contained 10,200 units of enzyme per ml with a specific activity of 13.2. One ml of the above extract was placed in a two cm wide starch column in an initial trench about one cm in length. The column was allowed to develop for 980 min at 370 v and 11 milliamperes. Then it was cut into one cm blocks, each of which was eluted by shaking with 4 ml of H_2O for one hour. The eluates were analyzed then for enzyme and protein. The results obtained are described in figure 1 in which are plotted the units of enzyme and μg of protein per ml of eluate as a function of the distance from the starting trench. It will be noted that a large amount of protein in such extracts moves very slowly. The evidence available indicates that much of this slow moving protein is associated with nucleic acid. The enzyme moves fairly rapidly and as a relatively narrow peak. The specific activity of the enzyme in the peak region is 360 and is between 330 and 400 for the two cm on either side of the peak. These values are to be compared with 13.2 for the input material. Thus, one run can effect an approximately 30-fold purification. Eighty per cent of the input enzyme can be recovered readily in any given run.

The effect of repeated successive runs on the purification of the enzyme may now be examined. Cells were induced to form enzyme much as in the condition described above. Melibiose (0.001 M) was the inductor, and the cells were growing logarithmically for a period equivalent to one division during the induction.

Extracts were prepared by sonic disintegration as described previously, and after removal of cell debris, the enzyme was partially purified and concentrated by the ammonium sulfate fractionation procedure detailed under Methods. The initial trench accommodated 4.5 ml of the concentrated enzyme preparation. The column was allowed to develop for 1,008 minutes at 245 v and 15 ma. The enzyme peak was located and

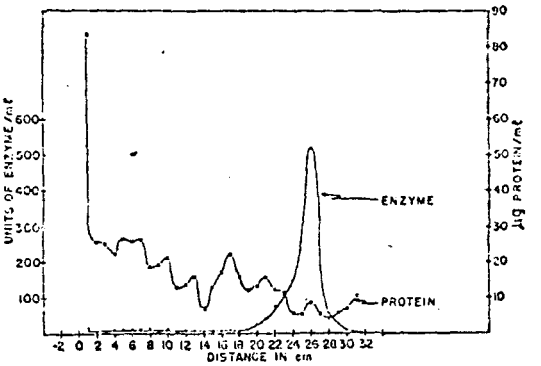

Figure 1. Distribution of enzyme and protein along the starch column in a first electrophoretic run at 370 v and 11 milliamperes for 980 minutes. At the pH employed (8.5) the enzyme travels towards positive electrode.

the specific activity of the enzyme in the peak region determined by examining a ½ cm strip removed from the column. Once located, the peak region of enzyme in the starch column was cut out, placed into an initial trench of appropriate size in a fresh column, and the process repeated. Table 1 summarizes the results obtained in four successive runs. It will be noted that the specific activity of the initial preparation was 45 and that the first run gave an enzyme preparation at the peak with a specific activity of 976 corresponding to a 20-fold increase. As is indicated by the results of run no. 4, the maximal purification was achieved by run no. 3.

Uniformity of protein labeling. The purpose of the present experiments required comparison between the specific radioactivity of the enzyme and the nonenzymatic protein in the cell. Such a comparison is meaningful only if the nonenzymatic proteins are uniformly labeled. Initial uniformity in the labeling is assured readily by employing an inoculum which is small relative to the final yield of culture when growing up cells in the C^{14}-lactate. The assumption that growth of such cells in an identical medium which was unlabeled would result in a uniform dilution of all existent proteins was tested directly. Cells uniformly labeled to the extent of 101 counts per min per μg N were prepared by growth in C^{14}-lactate. The cells were washed thoroughly with cold medium containing 0.3 per cent of unlabeled L-lactate, resuspended in the same medium, and allowed to go through approximately one division. The cells then were har-

TABLE 1

Repeated purifications of peak regions

Culture allowed to go through one division in the presence of 0.001 M melibiose; cells washed, disintegrated, and debris removed; enzyme concentrated by two ammonium sulfate precipitations (1st at 65 per cent and 2nd at 52 per cent saturation); residual ammonium sulfate removed by dialysis. This preparation was run four successive times on the starch columns, the peak region being removed in each case. Activities in peak regions are given in each case.

RUN NO.	DURATION	VOLTS	MILLIAMPERES	POSITION OF PEAK IN CM FROM ORIGIN	SPECIFIC ENZYME ACTIVITY	PORTION REMOVED FOR RE-RUN
	min				*mμM ONPG*/ min/μg N*	*cm*
Initial preparation	—	—	—	—	45	—
1	1,008	245	15.0	16	976	15.5–17.5
2	1,059	245	15.5	17	1,360	18.5–20
3	1,036	245	13.0	15	1,950	13.5–15.5
4	1,030	250	10.5	15	1,890	—

* ONPG—*o*-nitrophenyl-β-D-galactoside.

vested, washed, and lysed by sonic treatment. After removal of cell debris by centrifugation, and without any prior fractionation, the extract was placed in the starch column and allowed to develop at 400 v and 11 milliamperes for a period of about 15 hr. Subsequently, the column was cut up into portions one cm in length, and each section eluted separately. The proteins in the eluates were precipitated and purified by the methods described, and the specific radioactivities determined. The mean specific radioactivity of the protein (plus and minus two standard deviations) of 30 such starch sections was found to be 48.2 ± 4.3 counts per min per μg N. All the values were encompassed within two standard deviations of the mean indicating no statistical difference in the degree of dilution experienced by the proteins which were so isolated and examined.

The maximal specific enzyme activity. Data obtained in the course of repeated purification of enzyme on the starch columns suggested that the maximal specific activity of the enzyme was in the neighborhood of 1,900 mμM per min per μg N under the conditions of our assay. Comparison with previously published (Cohn and Monod, 1951; Lester, 1952; Kuby and Lardy, 1953) figures on specific activities indicates that the starch column procedure yields preparations of higher purity. It was of interest, particularly in view of its usefulness as a means of estimating enzyme protein, to obtain further information on the validity of the maximal figure. The use of specific precipitation of radioactively labeled enzyme with purified antiserum suggested itself as a possible method for another check on this figure. Cohn and Torriani (1952) and Cohn (1953) have provided the conditions under which such precipitations can be carried out. They have demonstrated also that the enzyme in the insoluble antigen-antibody complex expresses full activity. Were the enzyme in such a complex uniformly labeled with a radioactive tracer and its specific radioactivity (in counts per min per μg N) known, the amount of enzyme protein in the precipitate could be determined readily. With the latter figure and the enzyme activity, the specific enzyme activity would be known. The efficiency of such specific precipitation in removing extraneous material is illustrated by the work of Cohn and Hogness (Monod and Cohn, 1953) as well as the results reported in the present paper.

Uniformly labeled cells were grown with aeration in synthetic medium containing C^{14}-L-lactate as the sole carbon source. During the growth, melibiose (0.001 M) was introduced as an inductor. Such conditions would insure uniformity of labeling of all the proteins of the cell, including the β-galactosidase. The cells were harvested, extracts prepared, and enzyme concentrated as previously described. An aliquot was placed on a starch column and allowed to develop. The enzyme peak was located and re-run in a fresh column. The remainder of the first column was cut up into one cm sections which were eluted, and the average specific radioactivity of the proteins determined. The mean ±2 standard

errors was found to be 155 ± 3.9 counts per min per μg N, and no significant deviations from it were observed. After several successive runs on the starch columns the enzyme peak region was eluted. The eluate contained 583 enzyme units per ml.

One ml of this eluate was subjected to precipitation with β-galactosidase antiserum purified and prepared as described under Methods. To increase the specificity of the precipitation it was carried out in the presence of unlabeled extract from noninduced cells, which insures that all nonenzymatic antigenic components would be in the region of antigen excess. The reaction mixture had the following components: 1 ml of eluate, 1 ml of antiserum, 1 ml of unlabeled noninduced extract, 0.2 ml of 10 per cent NaCl, 0.2 ml of pH 7.5 phosphate buffer. The antiserum was added last. The reaction mixture was held for 96 hours at 4 C, and the precipitate collected by centrifugation and washed three times with cold 0.02 M phosphate buffer at pH 7.0. The precipitate was resuspended then with homogenization in 0.9 ml of the same buffer.

Both the supernate and the resuspended precipitate were assayed for enzyme. None was found in the former, and 96 per cent of the input enzyme activity was accounted for in the antigen-antibody precipitate.

Radioactive counts on a 0.4 ml aliquot of the resuspended precipitate yielded a value of 49.8 ct per ml. From the average specific radioactivity of the cellular protein we find that the antigen-antibody complex contains 0.32 μg N of cellular protein per ml. We find, assuming that all the input enzyme material is in the complex, that the specific enzyme activity is 1,820 mμM per min per μg N, in reasonable agreement with the previous "maximal" figure of 1,900.

Synthesis of enzyme in labeled cells suspended in unlabeled medium. Uniformly labeled cells of *E. coli* were obtained by growth of a culture in one liter of synthetic medium containing 0.1 per cent L-lactic acid with a specific radioactivity of 1.6×10^3 ct per min per μM. The culture was started by a small inoculum from a stock slant and allowed to incubate with vigorous aeration at 37 C for 15 hours. The cells were harvested by centrifugation and washed twice with cold medium to remove residual radioactivity and resuspended in 5 ml of H_2O. The latter then was added to 4 liters of synthetic medium previously equilibrated to 37 C and containing 0.2 per cent of unlabeled L-lactic acid as the sole carbon source. The suspension was incubated with vigorous aeration at 37 C. Samples were removed at 10 min intervals for optical density determinations to ascertain the time of onset of logarithmic growth. Cultures so treated rarely have lag periods exceeding 10 minutes. Inductor in the form of melibiose (0.001 M) was added after logarithmic growth was assured and the incubation continued in the presence of inductor for a period corresponding to 0.9 divisions. Samples were removed periodically for both optical density determinations and enzyme assay.

At the end of the period indicated, the cells were harvested in a Sharples centrifuge, washed with 90 ml of H_2O, resuspended in 50 ml of H_2O, and sonicated. The enzyme in the extract was partially purified and concentrated by ammonium sulfate fractionation as described previously, and further purification achieved on a starch column. Table 2 describes the progress of the purification during successive runs of the peak region. This particular experiment was terminated after the fourth run since there was not enough material left to permit adequate estimations of protein content, enzyme, and radioactivity in a fifth run. The data are given in terms of the specific enzymatic activity and specific radioactivity of the peak region. The latter is compared with the specific radioactivity of the average protein.

It is evident from an examination of the data that as the purification of the enzyme progresses the specific radioactivity falls. The data detailed in table 2 support the statement that less than 9.1 per cent of the carbon of the newly formed enzyme molecules can be derived from any preexisting carbon in the cell.

In other similar experiments, analogous results were obtained, the degree to which the enzyme could be freed of radioactivity being about the same and independent of the length of the induction. The purification and analytical methods employed demand a minimal amount of enzyme for the successful completion of the experiment. This lower limit is such as to preclude experiment involving inductions, running much below 0.5 divisions, even with the use of the more powerful inductors such as *n*-butyl-β-D-galactoside.

The question arises whether the somewhat less than 10 per cent radioactive carbon found in the

TABLE 2

Specific radioactivity of enzyme synthesized by C^{14} labeled cells suspended in unlabeled medium

Cells uniformly labeled by growth in C^{14}-lactate induced (0.001 M melibiose) to form enzyme in unlabeled medium for a period of 0.9 divisions. Enzyme, isolated and concentrated as previously described, was run four successive times in starch columns. Specific radioactivity (ct/min/μg N) and specific enzyme activity (mμM of ONPG/min/μg N) of the input material and the peak regions of enzyme activity in successive runs are given. Column 4 is calculated by means of the "maximal" specific activity value of 1,900. Column 6 is calculated by subtracting corresponding values in column 5 from 100.

(1) RUN NO.	(2) SPECIFIC RADIOACTIVITY (ψR)	(3) SPECIFIC ENZYME ACTIVITY	(4) % PURIFICATION FROM SPECIFIC ENZYME ACTIVITY	(5) 100 × $\frac{\psi R \text{ OF ENZYME PEAK}}{\psi R \text{ OF AVERAGE PROTEIN}}$	(6) % PURIFICATION FROM SPECIFIC RADIOACTIVITY
Initial preparation	46.1	—	—	—	—
1	21.5	1,030	54.3	46.6	53.4
2	12.9	1,320	69.5	28.0	72.0
3	6.8	1,620	85.3	14.7	85.3
4	4.2	1,800	94.7	9.1	90.9

enzyme purified by the starch column method represents a small, but measurable, proportionate utilization of preexisting carbon in the course of enzyme synthesis. Information pertinent to this issue may be obtained by comparing columns 4 and 6 of table 2. Column 4 represents the "percentage purification" achieved at each step calculated from the specific enzyme activity as compared with the maximal value of 1,900. Column 6 calculates the "percentage purification" from the relative specific radioactivity (column 5) on the assumption that the enzyme is completely unlabeled. The relatively good agreement between these figures suggests that the residual radioactivity is not enzymatic but is rather assignable to contaminating material. Such calculations, however, are not of sufficient sensitivity to be completely decisive at low levels. Therefore, immunological precipitation was used to decide this question.

Specific precipitation of partially purified enzyme with antiserum. The immunologic procedures described above were used. It may be noted that it was found, in agreement with Cohn and Monod (personal communication), that selective removal of enzyme protein with antiserum cannot be achieved in crude extracts. Preliminary partial purification is essential. In the present experiment this preliminary purification was attained by the starch column method. C^{14} labeled cells were prepared and induced to form enzyme while suspended in unlabeled lactate medium as described above. The induction was carried out in log phase for a period of 0.5 doublings in the presence of 0.001 M melibiose. The preparation of the extract was placed in the starch column, and the peak region of enzyme activity run three successive times on starch columns. On the third run, the eluate from the enzyme peak region contained a protein with a specific enzymatic activity of 1,300 mμM per min per μg N. Comparison with the maximal specific enzyme activity would indicate that 7 per cent of purity had been achieved. This value is in excellent agreement with what would be estimated from its specific radioactivity, assuming the enzyme protein was unlabeled. The protein contained label to the extent of 19.2 ct per min per μg N which is 30.1 per cent of the value of 63.7 ct per min per μg N found for the average nonenzymatic protein in the same cells.

The eluate, containing 5,000 units of enzyme per ml, was subjected to specific precipitation with purified β-galactosidase antiserum following the procedure described above. Again to insure the specificity of the precipitation it was carried out in the presence of unlabeled extract from noninduced cells, so that all nonenzymatic components would be in the region of antigen excess. The reaction mixture was held at 4 C for 48 hours, and the precipitate was collected by centrifugation. It was washed twice with 0.1 M phosphate buffer at 7.5 and resuspended with homogenization in 0.6 ml of H_2O. An aliquot of 0.4 ml was used for the determination of radioactivity and 0.05 ml for the assay of enzyme. All of the original activity contained in the eluate was accounted for in the antigen-antibody precipitate, which checked with the fact that no detectable enzyme was found in the supernate.

TABLE 3

Purification by specific precipitation with antiserum

One ml of starch column eluate was added to the following: 1.0 ml of unlabeled extract from noninduced cells, 0.2 ml of 10 per cent NaCl; 0.2 ml of 0.1 M phosphate buffer at pH 7.5. To the resulting mixture was added 1 ml of purified antiserum. The resulting precipitate was washed and prepared as described in the text. The aliquot measured for radioactivity contained 0.33×10^4 enzyme units. The counting was continued until 5,910 counts had accumulated (300 min). A background count over the same time period yielded 5,972 counts. The absolute error was determined from the relation $E = k\sqrt{N_B + N_S}$ where N_B is the background count, N_S the count of the sample, and k the probability constant. The latter was taken as 3.29, which yields the "999/1,000" error. The protein assignable to enzyme in the antigen-antibody precipitate was determined from the maximal enzyme specific activity value of 1,900 and the observed enzyme activity. The figures in the second row are calculated from the latter and the determined absolute error of counting. The chances are one in a thousand that the actual figures can be greater than those stated.

	COUNTS PER MINUTE PER 1×10^4 ENZYME UNITS	COUNTS PER MINUTE PER μG N (ψR)	$\frac{\psi R \text{ OF ENZYME}}{\psi R \text{ OF AVERAGE PROTEIN}} \times 100$
Before precipitation with antiserum....	176	19.2	30
After precipitation with antiserum....	<3.6	<0.69	<1.0

Table 3 summarizes the results obtained in the radioactivity measurements. Although all the enzyme was removed from the eluate by the specific precipitation, no detectable amount of radioactivity was found in the antigen-antibody complex. The sample count was not significantly different from background. It would appear that the residual activity found in enzyme preparations purified by the starch column procedure is due to small amounts of contaminating proteins. This type of specific precipitation was carried through a number of times on similar preparations and, in all cases, removed virtually all of the contaminating radioactivity.

On the basis of the maximal specific enzyme activity, the figures listed in table 3 indicate that less than one per cent of the carbon of a newly formed enzyme molecule comes from any preexisting carbon in the cell.

DISCUSSION

The experiments described in the present paper offer little support for the preexistence in the cell of any significant amount of precursor material, simple or complex, which is utilizable for the synthesis of new enzyme molecules. These results, taken together with the independently performed experiments of Cohn and Hogness (Monod and Cohn, 1953), make it difficult to avoid the conclusion that the induced synthesis of β-galactosidase is a virtually *de novo* process employing principally amino acids which are formed subsequent to the moment of the addition of the inducer.

A brief statement may be interposed here concerning protein turnover. It is obvious that had there been any extensive breakdown and resynthesis of protein in the course of the experiments reported here, a proportionate amount of label would have made its appearance in the β-galactosidase. No evidence was detected for such turnover. The same is true for the experiments of Cohn and Hogness described by Monod and Cohn (1953). In the latter experiments attempts were made to see whether unlabeled enzyme in S^{35} labeled cells would pick up label either in the presence or in the absence of inducer. No such interchange was observed.

Such observations would appear to pose the problem of whether the proteins of growing bacterial cells differ from those of adult mammalian tissue in which "turnover" can be detected. In part the difference is a spurious one, assignable to the markedly different time scales of the experiments performable with mice and men as compared with *E. coli*. A sample calculation should make the relevant issue clearer. We may take a half-life of 6 days (Borsook, 1953) as a sufficiently representative figure for the turnover rate of a relatively rapidly synthesizing mammalian tissue. This would yield a decay constant in hours of -0.0048. A comparatively long term experiment with *E. coli*, of the type we have described, would extend over a period of two hours. Consider one liter of an *E. coli* culture at 1×10^9 organisms per ml uniformly labeled to a level of 100 counts per min per μg protein N. If these proteins possessed the decay constant noted above, they would, in

the course of a two hour period, liberate one per cent of their radioactivity, or 1×10^4 ct per min. Assuming as seems reasonable, that all proteins being synthesized can participate equally in incorporating this radioactivity and that 0.1 per cent of the protein being formed in the two hour induction period is β-galactosidase, approximately 10 ct per min would be found in the entire enzyme fraction. This would be barely detectable even if a considerable portion of the enzyme formed was isolated in a pure state. On the basis of specific radioactivity, it would be just below the level of adequate measurement since it would be somewhat less than one ct per min per μg N.

It is clear from the above discussion that experiments on induced synthesis, performed in the manner described, might well miss turnover rates of levels characteristic of mammalian tissues. The data accumulated in the course of the present investigation would indicate that the protein breakdown rates in growing *E. coli* cells cannot have decay constants much greater than 0.005, and they may possibly be less. This is in good agreement with some recent results obtained by Anker (1954, personal communication) who followed the release of C^{14}-arginine from bacterial proteins in *E. coli* growing in a chemostat. The results indicate that less than one per cent of the arginine in protein was released over a period corresponding to a 3.1-fold increase in mass during the growth of an *E. coli* strain. Podolsky (1953) reports a somewhat higher figure for *E. coli* growing in a synthetic medium under somewhat different conditions. He finds that over a 30 hour period 8 per cent of the labeled arginine is lost, corresponding to a decay constant of 0.008.

One further fact may be noted. A simple calculation reveals that the specific rate of protein synthesis (mass/mass/unit time) is of the order 1,000 times faster in bacterial cells growing in log phase than that found, e.g., in the liver of the adult rat in nitrogen balance, the proteins of which have a half-life of about 6 days. We have seen nevertheless that the decay rate of bacterial proteins cannot be much greater than that corresponding to a 6 day half-life and is indeed in all probability less. This would appear to eliminate any interpretation of "turnover", or the "dynamic state" of proteins which seeks to link in a compulsory manner rates of protein breakdown and synthesis.

SUMMARY

Experiments have been designed to test the following two possibilities for the synthesis of β-galactosidase by *Escherichia coli*:

(a) Precursor + free amino acids → Active enzyme.

(b) Free amino acids → Active enzyme.

Enzyme synthesis was induced in cells uniformly labeled with C^{14} while they were suspended in unlabeled medium. Isolation and purification of the β-galactosidase synthesized revealed that less than one per cent of its carbon could have been derived from any cellular components existing prior to the moment of the addition of the inducer. These findings virtually eliminate any hypothesis which presupposes the preexistence of precursor material, simple or complex, convertible into enzyme.

The data further indicate that protein synthesis in the growing *E. coli* cell is virtually irreversible. Protein "turnover" even remotely approximating the synthetic rate could not be detected.

REFERENCES

BARKER, S. B., AND SUMMERSON, W. H. 1941 The colorimetric determination of lactic acid in biological material. J. Biol. Chem., 138, 535-554.

BORSOOK, H. 1953 Protein turnover and incorporation of labeled amino acids into tissue proteins *in vivo* and *in vitro*. Physiol. Revs., 30, 206-219.

COHN, M. 1952 Techniques and analysis of the quantitative precipitin reaction. In *Methods in medical research*. Vol. 5. Edited by A. C. Corcoran. The Year Book Publishers, Inc., Chicago, Illinois.

COHN, M. 1953 A note on the use of the antigen excess zone to reveal the existence of certain types of cross reactions in unidentified mixtures of antigens. J. Immunol., 70, 317-320.

COHN, M., AND MONOD, J. 1951 Purification et proprietes de la beta-galactosidase (lactase) d'*Escherichia coli*. Biochim. et Biophys. Acta, 1, 153-174.

COHN, M., AND TORRIANI, M. 1952 Immunochemical studies with the β-galactosidase and structurally related proteins of *Escherichia coli*. J. Immunol., 69, 471-491.

COHN, M., AND TORRIANI, A. 1953 The relationships in biosynthesis of the β-galactosidase and Pz-proteins in *E. coli*. Biochim. et Biophys. Acta, 10, 280-289.

CREMER, H. D., AND TISELIUS, A. 1950 Elektrophorese von Eiweis in Filtrierpapier. Biochem. Z., 320, 273-283.

DURRUM, E. L. 1950 A microelectrophoretic and microionophoretic technique. J. Am. Chem. Soc., 72, 2943-2948.

FREUND, J., AND BONANTO, W. V. 1944 The effect of paraffin oil, lanolin-like substances, and killed tubercle bacilli on immunization with diphtheria toxoid and *B. typhosum*. J. Immunol., 48, 325-334.

GIBBS, M., DUMROSE, R., AND ARCHER, F. 1952 *Biochemical preparation*. Vol. 2. Edited by E. G. Ball. John Wiley and Sons, N. Y.

HALVORSON, H. O., AND SPIEGELMAN, S. 1952 The inhibition of enzyme formation by amino acid analogues. J. Bacteriol., 64, 207-221.

HALVORSON, H. O., AND SPIEGELMAN, S. 1953*a* The effect of free amino acid pool levels on the induced synthesis of enzymes. J. Bacteriol., 65, 496-504.

HALVORSON, H. O., AND SPIEGELMAN, S. 1953*b* Net utilization of free amino acids during the induced synthesis of maltozymase in yeast. J. Bacteriol., 65, 601-608.

HUDSON, C. S., AND HARDING, T. S. 1915 The preparation of melibiose. J. Am. Chem. Soc., 37, 2734-2736.

KOPPEL, J. L., PORTER, J. C., AND CROCKER, B. F. 1953 The mechanism of the synthesis of enzymes. I. Development of a system suitable for studying this phenomenon. J. Gen. Physiol., 36, 703-922.

KUBY, S. A., AND LARDY, H. A. 1953 Purification and kinetics of β-d-galactosidase from *E. coli* strain K-12. J. Am. Chem. Soc., 75, 890-896.

KUNKEL, H. G., AND SLATER, R. J. 1952 Zone electrophoresis in a starch supporting medium. Proc. Soc. Exptl. Biol. Med., 80, 42-44.

KUNKEL, H. G., AND TISELIUS, A. 1951 Electrophoresis of proteins on filter paper. J. Gen. Physiol., 35, 89-118.

LANNI, F., DILLON, M. L., AND BEARD, J. W. 1950 Determination of small quantities of nitrogen in serological precipitates and other biological materials. Proc. Soc. Exptl. Biol. Med., 74, 4-7.

LEDERBERG, E. M. 1952 Allelic relationships and reverse mutation in *E. coli*. Genetics, 37, 469-483.

LEDERBERG, J. 1950 The β-D-galactosidase of *Escherichia coli*, strain K-12. J. Bacteriol., 60, 381-392.

LEE, N. D., AND WILLIAMS, R. H. 1952 Inhibition of adaptive formation of tryptophane peroxidase in rats by ethionine. Biochim. et. Biophys. Acta, 9, 698-699.

LESTER, G. 1952 The β-galactosidase of lactose mutants of *E. coli*, K-12. Arch. Biochem. and Biophys., 40, 390-401.

LESTER, G., AND BONNER, D. M. 1952 The occurrence of β-galactosidase in *Escherichia coli*. J. Bacteriol., 63, 759-769.

LOWRY, O. H., ROSEBROUGH, M. J., FARR, A. C., AND RANDALL, R. J. 1951 Protein measurement with the folin-phenol reagent. J. Biol. Chem., 193, 265-275.

MONOD, J., AND COHN, M. 1952 La biosynthese induite des enzymes. Advances in Enzymol., 13, 67-119.

MONOD, J., AND COHN, M. 1953 Sur la mecanisme de la synthese d'une proteine bacterienne. Symposium on microbial metabolism. IV. International Congress Microbiological Congress, Rome.

MONOD, J., COHEN-BAZIRE, G., AND COHN, M. 1951 Sur la biosynthese de la β-galactosidase (lactase) chez *E. coli* la specificite de L'induction. Biochim. et Biophys. Acta, 7, 585-599.

MONOD, J., PAPPENHEIMER, A. M., AND COHEN-BAZIRE, G. 1952 La cinétique de la biosynthése de la β-galactoside chez *E. coli* considere comme fonction de la croissance. Biochim. et Biophys. Acta, 9, 648-660.

NICHOL, J. C., AND DEUTSCH, H. F. 1948 Biophysical studies of blood plasma proteins; separation of γ-globulin from the sera of various animals. J. Am. Chem. Soc., 70, 80.

PODOLSKY, R. J. 1953 Protein degradation in bacteria. Arch. Biochem. and Biophys., 45, 327-340.

RICKENBERG, H. V., YANOFSKY, C., AND BONNER, D. M. 1953 Enzymatic deadaptation. J. Bacteriol., 66, 683-687.

SEIDMAN, M., AND LINK, R. P. 1950 Substituted salicylaldehydes and derivatives. J. Am. Chem. Soc., 72, 4324.

TAYLOR, E. S. 1947 The assimilation of amino acids by bacteria. 3. Concentration of free amino acids, in the interval environments of various bacteria and yeast. J. Gen. Microbiol., 1, 86-90.

USHIBA, D., AND MAGASANIK, B. 1952 Effects of auxotrophic mutations on the adaptation of inositol degradation in *Aerobacter aerogenes*. Proc. Soc. Exptl. Biol. Med., 80, 626-632.

WOOD, H. G., LIFSON, AND LORBER, V. 1945 The position of fixed carbon in glucose from rat liver glycogen. J. Biol. Chem., 159, 475-489.